PHOSPHOMONOESTERASE ACTIVITY IN HEPATIC TISSUES OF THE MOUSE*

by

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A series of investigations has been conducted concerning the chemical and enzymatic characterization of mouse hepatic tissues[1,2,3,4]. As an extension of these studies, phosphomonoesterase within normal and regenerating mouse hepatic tissues was investigated. An extensive literature exists concerning the enzymes associated with the dephosphorylation of a variety of organic phosphates and has been the subject of a number of reviews[5,6,7,8]. Assuming the presence of numerous separate phosphomonoesterases within mouse hepatic tissue, the present study was restricted to the enzymes (or enzyme) capable of hydrolyzing monophenyl phosphate in both an acid as well as alkaline medium.

The investigations to be reported were grouped into three separate general studies. The first included the development of adequate quantitative methods for the accurate assay of phosphomonoesterase within fresh tissue homogenates. The second study was concerned primarily with the measure of phosphomonoesterase levels within normal adult as compared to regenerating hepatic tissues of the mouse at various intervals during the restoration process. The final study involved a series of experiments attempting to localize the sites of phosphomonoesterase within tissue fractions isolated by differential centrifugation techniques from normal adult and regenerating mouse liver.

MATERIALS AND METHODS

Preparation of tissues

Livers from 18 hour fasted male Strain A mice between 2–3 months of age were used in all of the experiments to be reported. The livers were removed from mice under ether anaesthesia with special care to allow maximum drainage of blood. The excised, bled livers were weighed and ground in all-glass homogenizers (Scientific Glass Co.) with glass distilled water for all experiments except the centrifugation studies for which a solution of 0.88 *M* sucrose was substituted.

Liver regeneration was induced within one group of mice by surgical removal of approximately two thirds of this organ. Mice of approximately equal weight (21 ± 1 gram) were selected for these experiments. The techniques employed were similar to those used by HIGGINS AND ANDERSON to induce liver regeneration in rats[9]. Mice were sacrificed at intervals of ½, 2, 3, 5 and 8 days following the surgical operation. The mice were allowed Purina Laboratory Chow and water *ad libitum* up to 18 hours prior to sacrifice, at which time the food alone was removed.

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In those experiments employing centrifugation techniques for the separation of cellular constituents, procedures similar to those proposed by HOGEBOOM, SCHNEIDER, AND PALLADE[10] were incorporated. Using liver homogenates (1:10) prepared in 0.88 *M* sucrose solution, the nuclear fraction was separated by spinning for 30 minutes at 800 × *g* (International Clinical Centrifuge). The large granule fraction (mitochondria) was separated by spinning the cytoplasmic extract at approximately 23,000 × *g* for 20 minutes (International Refrigerated Centrifuge, Model PR-1 with high speed attachment). The remaining supernatant fluid was spun for 2 hours at approximately 40,000 × *g* (Sorvall Co. Type SS-2 Centrifuge) for the separation of the submicroscopic fraction (microsomes) from the final supernatant liquor. The sedimented nuclear, mitochondrial and microsomal fractions were resuspended without further washing with 0.88 *M* sucrose. All fractions were diluted to a volume comparable with the original homogenate (1:10). Temperatures were maintained as close to 0° C as possible throughout the centrifugation procedure with the aid of a walk-in cold box in which the non-refrigerated centrifuges were placed. The prepared fractions were diluted as required and assayed together immediately following the isolation of the final fractions. Nitrogen analyses were performed on each of the fractions using an application of the micro-KJELDAHL procedure[11].

Substrate purification

Commercial preparations of disodium phenylphosphate were purified from contaminating decomposition products prior to their use. The purification was accomplished by dissolving the phenylphosphate in a minimum volume of methanol, leaving a large proportion of the contaminating inorganic phosphate behind as an insoluble residue. The phenylphosphate was precipitated from the methanol solution by the addition of an equal volume of ethyl ether. The precipitate was washed several times with ethyl ether and dried. Little inorganic phosphate or free phenol was found in the purified preparations. Substrate solutions were prepared at frequent intervals and always titrated to appropriate hydrogen ion concentration prior to use in view of the inherent buffering capacity of this compound.

General procedure

Standard procedures were adopted for the routine study of the liver phosphomonoesterases. All reactions were carried out in 25 ml Erlenmeyer flasks. Reaction mixtures were maintained at a 3 ml volume containing tissue, substrate, magnesium ions and buffer. Conventional WARBURG constant temperature water baths set for 37° C equipped with special shaking racks were employed for all incubations, assuring good mixing throughout the reaction period. Following the designated incubation time, 7 ml cold 10% trichloroacetic acid solution was added to each flask and the contents filtered using Whatman No. 5 paper. Aliquots of the filtrate were analyzed for inorganic phosphorus by the FISKE AND SUBBAROW method[12]. Appropriate tissue and substrate blanks were carried for all of the enzyme analyses.

EXPERIMENTAL

1. *Quantitative methods for the accurate estimation of phosphomonoesterase activity within tissue homogenates*

Aqueous extracts of autolyzed liver have been described[13, 14, 15] as containing enzymes capable of hydrolyzing monoesters of phosphoric acid with both acid and alkaline p_H optima. From these findings, the presence of two enzymes referred to as "acid" and "alkaline" phosphatase has been concluded. Using homogenate preparations of mouse liver, the effect of hydrogen ion concentration on the dephosphorylation of monophenylphosphate was investigated (Fig. 1). In the study presented, the results obtained on homogenized mouse liver immediately following preparation have been compared with findings on the same sample allowed to stand for 24 hours at near 0° C. It is apparent from these results that a sharp maximum in activity occurs at or near p_H 5.5. Although a second p_H optimum in the extreme alkaline regions was not apparent, a minor secondary peak at p_H 7.5 was observed. It was of some interest to find that liver tissue on standing showed a marked reduction in phosphomonoesterase activity only in the acid solutions, with little loss observed at the alkaline levels. These results

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might suggest the presence of separate enzymes active in the acid and alkaline regions. Accurate methods for the quantitative estimation of phosphomonoesterase activity at the acid optimum (p_H 5.5) as well as in the extreme alkaline region (p_H 9.2) were devised for purposes of subsequent investigations.

The procedure adopted for the quantitative assay of phosphomonoesterase activity at p_H 5.5 and 9.2 was based upon the results of preliminary investigations dealing with optimum substrate concentration, effects of magnesium ions and appropriate incubation periods. It was considered desirable to standardize procedures so that assays at p_H 5.5 and 9.2 might be conducted using identical conditions in all respects excluding the buffers involved.

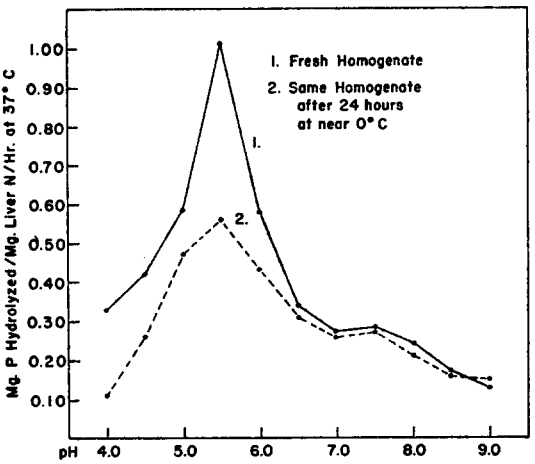

Fig. 1. Effect of hydrogen ion concentration on phosphomonoesterase activity in fresh and aged mouse liver homogenate. Reaction mixtures contained 0.16 mg liver N and a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* buffer (acetate to p_H 6.0, veronal from p_H 6.5–9.0) in a final volume of 3 ml

Substrate concentration. The effect of substrate concentration on phosphomonoesterase activity at p_H 5.5 and 9.2 was investigated (Fig. 2). At substrate levels less than 250 micromoles at p_H 9.2 and 188 micromoles at p_H 5.5, phosphomonoesterase activity remains as a function of substrate concentration. At those substrate levels tested at or above 250 micromoles, however, there remains little influence of substrate concentration upon enzymatic activity (zero order reaction rate). From these findings it was concluded that substrate concentrations between 250–500 micromoles should be optimal for the assay of phosphomonoesterase activity at both p_H 5.5 and 9.2 within the experimental conditions designated.

Magnesium concentration. Numerous experiments were conducted in an attempt to define an optimum concentration of magnesium ions for maximum activation of liver phosphomonoesterase at both p_H 5.5 and 9.2. Magnesium concentrations ranging from 0.012–0.24 molar (final concentration) were tested using homogenates of single liver samples. Considerable variation was observed between liver samples with respect to the extent of activation induced by magnesium ions; however, in all cases at least some activation was apparent at both p_H 5.5 and 9.2. Variations in activation were attributed to the relative presence or absence of natural activating

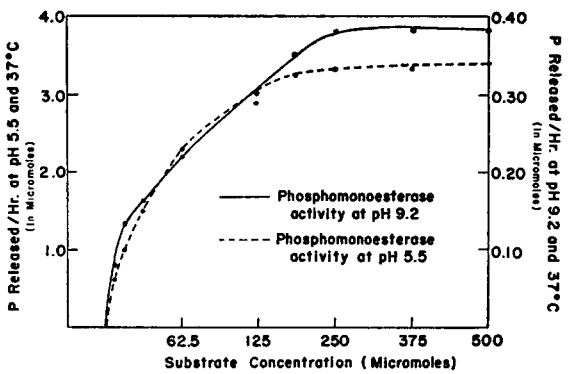

Fig. 2. Effect of substrate concentration on phosphomonoesterase activity. Reaction mixtures contained in addition to substrate 2.5 mg fresh liver, a final concentration of 0.12 *M* Mg ions and 0.05 *M* buffer (acetate p_H 5.5, veronal p_H 9.2) in a final 3 ml volume

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agents inherent within the different liver samples investigated. Little inhibition of phosphomonoesterase activity was found even in the presence of a considerable excess of magnesium ions. Although in excess to that generally required, a concentration of 0.12 molar magnesium ions was selected for subsequent enzyme assays to insure optimum phosphomonoesterase activity at both p_H 5.5 and 9.2 for any given liver sample. Activation by magnesium was found to be greater at p_H 9.2 than 5.5.

Incubation time. The relationship between phosphomonoesterase activity and incubation time was investigated in the presence of an optimum excess of substrate and magnesium (Fig. 3). It is apparent that a strict proportionality between enzyme activity and incubation time is maintained for a period somewhat exceeding one hour at both p_H 5.5 and 9.2 under these experimental conditions. Subsequent to this time, the reaction rate no longer remains constant but rather begins to decline. The extent to which this decline might be attributed to the possible gradual inactivation of enzyme was investigated by pre-incubating liver tissue for varying lengths of time prior to testing for enzymatic activity (Fig. 4). These results indicated little loss in enzyme activity at p_H 9.2 after as much as two hours pre-incubation of liver tissue at 37° C. On the other hand, losses in phosphomonoesterase activity at p_H 5.5 were evident after only 15 minutes pre-incubation with approximately a 50% reduction in activity following two hours. The marked decreases observed in enzyme activity at p_H 5.5 following the pre-

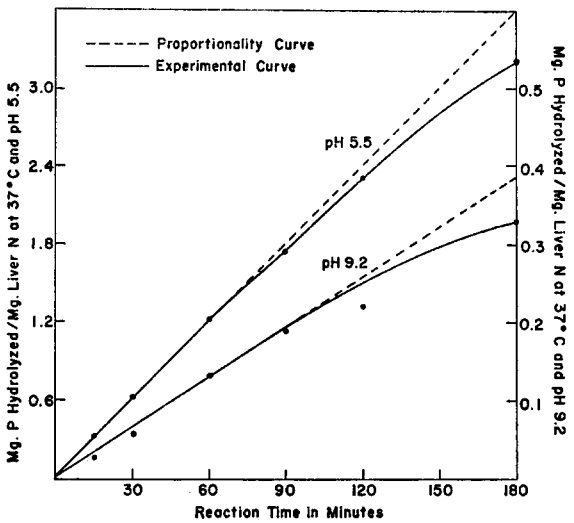

Fig. 3. Enzyme activity and incubation time. Reaction mixtures contained 2.5 mg fresh liver and a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* buffer (acetate p_H 5.5, veronal p_H 9.2) in a final 3 ml volume

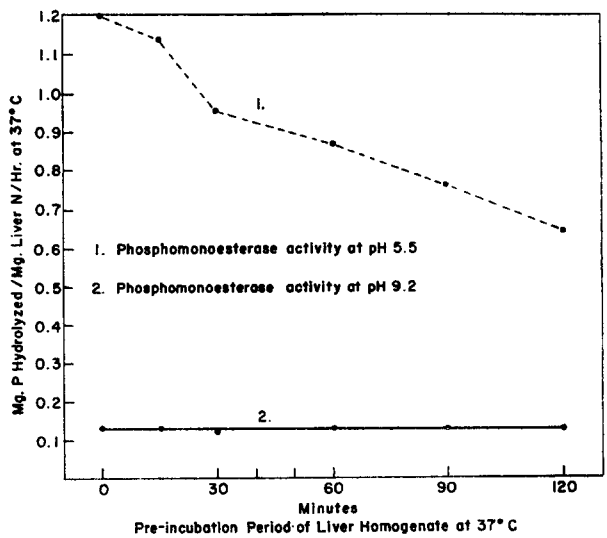

Fig. 4. Effect of pre-incubating liver tissue at 37° C on phosphomonoesterase activity. Following the specified pre-incubation periods, the liver tissues were assayed for enzyme using reaction mixtures containing 2.5 mg wet wt. liver and a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* buffer (acetate p_H 5.5, veronal p_H 9.2) in a final 3 ml volume

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incubation periods were not surprising in view of the results presented in Fig. 1; however, a discrepancy appears to exist between these results and the findings summarized in Fig. 3. In this study (Fig. 3) the decreases observed in enzyme activity with progressive incubation past the one-hour period were not of the order that one might predict from the pre-incubation studies, suggesting the presence of a protective influence of the added substrate on the enzyme. The differences observed in the effect of pre-incubating homogenates of liver tissue on phosphomonoesterase activity when determined at p_H 5.5 and 9.2 was considered to represent the presence of separate enzymes, one readily inactivated on standing (in the absence of substrate) and the other extremely stable.

Tissue concentration. A further consideration in attempting to define optimum conditions for the accurate assay of phosphomonoesterase activity within liver tissue involved the effect of tissue concentration on enzyme activity. The results of a representative experiment are shown in Table I. From these results it was concluded that with the experimental conditions devised, enzyme activity could be directly related to tissue concentration over an especially wide range of tissue levels at p_H 5.5 and a somewhat narrower range at p_H 9.2. The results of this study, therefore, afforded the basis for subsequent investigations requiring an accurate measure of phosphomonoesterase levels within mouse liver tissues.

TABLE I

PHOSPHOMONOESTERASE ACTIVITY AND TISSUE CONCENTRATION

mg fresh* liver assayed	Phosphomonoesterase** activity at p_H 5.5		Phosphomonoesterase** activity at p_H 9.2	
	Found (μg P)	Calculated per g fresh liver (mg P)	Found (μg P)	Calculated per g fresh liver (mg P)
1.00	39.8	39.8	5.15	5.15
1.25	49.8	39.8	6.33	5.06
1.67	67.0	40.2	8.28	4.96
2.50	102	40.8	12.5	5.00
5.00	205	41.0	24.5	4.90
7.50	309	41.2	36.0	4.80
10.0	390	39.0	46.1	4.61
20.0	768	38.4	77.0	3.85

* Represents aliquots removed from a homogenate of 5 mouse livers

** Enzyme activity determined for a period of 1 hour at 37° C. Reaction mixtures contained in a final 3 ml volume; tissue as designated in a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate, 0.05 *M* buffer (acetate p_H 5.5 and veronal p_H 9.2)

2. *Phosphomonoesterase activity in normal and regenerating hepatic tissues of the mouse*

In attempting to assign some role to the liver phosphomonoesterases, investigations have been reported regarding their presence within normal adult, as compared to regenerating, hepatic tissues. Using entirely different techniques, one group of investigators[16] reported little differences, while others[17, 18] concluded from their results that regenerating hepatic tissues contained increased levels of phosphomonoesterase as compared to normal adult liver. In view of the conflicting nature of these reports, it

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was considered desirable to re-investigate the problem in somewhat greater detail. In a previous report[4] it was concluded that liver regeneration induced in mice by a single feeding of carbon tetrachloride was accompanied by increases in phosphomonoesterase activity at p_H 9.2, with little change in enzyme levels when determined at p_H 5.5. The present investigation was concerned with the determination of phosphomonoesterase levels within fresh homogenates of adult normal and regenerating hepatic tissues of the mouse. Liver regeneration was induced by surgical removal of approximately two-thirds of this organ.

Phosphomonoesterase activity of normal adult mouse liver. Applying the assay procedures described in the previous section, phosphomonoesterase activity was determined at p_H 5.5 and 9.2 on liver homogenates of normal adult fasted mice (Table II). In view of the labile nature of enzyme determined at p_H 5.5, assays were performed on total liver homogenates immediately following their preparation. From the results obtained on 10 separate liver samples it was found that phosphomonoesterase activity at p_H 5.5 was 8–9 times greater than at p_H 9.2. No constant ratio between enzyme activity at p_H 5.5 and 9.2 was evident with considerable variation apparent in enzyme activity among the separate samples investigated.

TABLE II

PHOSPHOMONOESTERASE ACTIVITY OF NORMAL ADULT FASTED MOUSE LIVER DETERMINED AT p_H 5.5 AND 9.2

Mouse number	Weight of mouse (g)	Weight of liver (g)	mg N/g wet liver	Phosphomonoesterase activity at p_H 9.2*		Phosphomonoesterase activity at p_H 5.5*	
				Per g wet liver	Per mg Nitrogen	Per g wet liver	Per mg Nitrogen
1	21	1.19	36.4	4.37	0.120	53.4	1.47
2	21	1.20	35.3	4.22	0.120	47.6	1.35
3	21	1.07	36.7	6.22	0.170	38.8	1.06
4	22	1.12	36.3	4.22	0.116	51.8	1.43
5	21	1.10	36.4	4.71	0.130	43.4	1.19
6	22	1.22	34.2	5.54	0.162	35.2	1.03
7	21	1.12	38.2	5.58	0.146	32.2	0.84
8	22	1.06	34.8	5.25	0.151	40.0	1.15
9	21	1.00	38.6	6.24	0.161	50.0	1.30
10	20	0.98	38.6	4.60	0.119	40.2	1.04
Av.	21	1.11	36.6	5.10	0.139	43.3	1.19

* Phosphomonoesterase activity reported in terms of mg phosphorus hydrolyzed per hour at 37° C. All assays were conducted in duplicate on two levels of tissue (2.5 and 5.0 mg fresh liver) with average values recorded. All duplicate analyses were found to agree within 5% when computed in terms of per unit of tissue. Besides tissue, the reaction mixtures contained a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* buffer (acetate p_H 5.5, veronal p_H 9.2) in a final volume of 3 ml.

Phosphomonoesterase levels in regenerating liver. The preparation of tissues utilized in this study has been previously described under METHODS. Experiments were designed to follow possible alterations in phosphomonoesterase activity within regenerating liver tissue by sacrificing animals at various intervals subsequent to partial hepatectomy (Table III). It was found from these studies that even after 8 days the liver weights

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were not yet back to normal. Nitrogen values indicated an accumulation of large amounts of non-protein material (identified for the most part as neutral fat) within the regenerating livers soon after partial hepatectomy. From these results it was concluded that a truer assay of liver restoration might perhaps be approached by determining the amount of nitrogen rather than weight of tissue present. Enzyme activities were computed both on the basis of wet weight of liver and liver nitrogen, for purposes of comparison. The latter unit was considered of greater significance in view of the large quantities of lipid present especially in the early stages of liver regeneration. Although little change in phosphomonoesterase activity was evident at p_H 5.5, a marked increase in enzyme activity was found at p_H 9.2, reaching a maximum on the second day following partial hepatectomy. Similar results were obtained previously[4] on regenerating mouse liver following carbon tetrachloride administration. Increases in phosphomonoesterase activity within regenerating liver cell nuclei as detected by histochemical techniques[19, 20, 21] have been previously reported by others[17, 18]. Increases within rat liver cell nuclei following 78 hours regeneration of phosphomonoesterase active in alkaline media have been considered by some investigators[18] as evidence in support of their theories concerning a possible relationship between this enzyme and desoxypentose nucleic acid synthesis. Such a relationship might be questioned in view of other evidence[22]

TABLE III

PHOSPHOMONOESTERASE ACTIVITY IN REGENERATING MOUSE LIVER AT VARIOUS INTERVALS SUBSEQUENT TO PARTIAL HEPATECTOMY

Days following partial hepatectomy	No.* of mice	Liver weights (g)	mg N/g wet liver	Phosphomonoesterase activity p_H 9.2**		Phosphomonoesterase activity p_H 5.5**	
				Per g wet liver	Per mg Nitrogen	Per g wet liver	Per mg Nitrogen
Controls	10	1.11 (0.98–1.22)	36.6 (34.2–38.6)	5.10 (4.22–6.24)	0.139 (0.119–0.170)	43.3 (32.2–53.4)	1.19 (0.84–1.47)
½	5	0.59 (0.53–0.67)	25.8 (23.3–28.2)	3.90 (3.36–4.69)	0.150 (0.142–0.169)	23.7 (19.4–28.8)	0.92 (0.70–1.02)
2	5	0.67 (0.60–0.70)	25.9 (21.5–30.7)	9.38 (7.87–12.3)	0.362 (0.256–0.464)	32.1 (21.2–42.2)	1.24 (0.99–1.63)
3	5	0.75 (0.72–0.77)	28.0 (22.9–31.1)	7.58 (5.82–8.67)	0.271 (0.216–0.341)	28.4 (22.0–35.4)	1.01 (0.80–1.38)
5	5	0.88 (0.84–0.93)	32.5 (31.5–34.1)	8.18 (6.11–13.3)	0.252 (0.180–0.421)	37.0 (34.4–40.6)	1.14 (1.07–1.29)
8	4	0.98 (0.82–1.15)	34.7 (32.9–37.2)	5.35 (4.20–6.40)	0.154 (0.157–0.172)	30.4 (25.6–36.0)	0.88 (0.78–0.98)

* Mice were selected of approximately equal weights (22 ± 1 gram) prior to partial hepatectomy. All mice were sacrificed after an 18 hour fast. All values recorded for the control mice represent summaries from Table II.

** Phosphomonoesterase activity computed on the basis of mg phosphorus hydrolyzed per hour at 37° C. All assays were conducted on two levels of tissue (2.5 and 5.0 mg fresh tissue). All reaction mixtures contained in addition to tissue a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* buffer (acetate p_H 5.5, veronal p_H 9.2) in a final volume of 3 ml. Appropriate substrate and tissue blanks were carried for each analysis.

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indicating that desoxypentose nucleic acid synthesis occurs within 24 hours subsequent to partial hepatectomy in the rat. The extent to which the increases observed in phosphomonoesterase activity at p_H 9.2 might be attributed to the liver injury following surgical removal of a large part of this organ could not be determined.

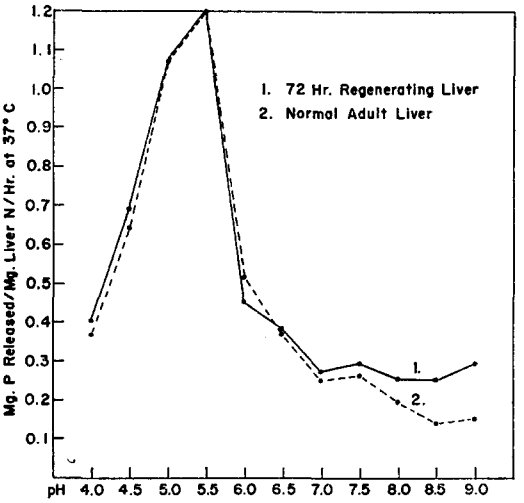

Fig. 5. Comparison of phosphomonoesterase activity and hydrogen ion concentration between normal adult and regenerating hepatic tissues of the mouse. Reaction mixtures contained 5 mg fresh liver tissue and a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* buffer (acetate to p_H 6.0, veronal from p_H 6.5–9.0) in a final 3 ml volume

p_H and phosphomonoesterase activity of regenerating liver. The relationship between hydrogen ion concentration and phosphomonoesterase activity within regenerating and adult hepatic tissues of the mouse was compared over a wide p_H range (Fig. 5). The measurements were made at the same time under identical conditions using pooled tissues from five mice for each group. The regenerating tissues were removed approximately 72 hours following partial hepatectomy. By computing phosphomonoesterase activity on the basis of tissue nitrogen, little difference in the shape of the two curves is apparent on the acid side of neutrality; however, with increasing alkalinity, regenerating tissue shows a gradual increase in enzyme activity over normal liver.

3. *Distribution of phosphomonoesterase activity within fractions prepared from normal and regenerating mouse liver by differential centrifugation*

Attempts to localize sites of enzymatic activity within cellular structures have led to the development of a number of different techniques. The two principal methods of approach include histochemical techniques as developed primarily by GOMORI[23] and the application of differential centrifugation procedures as introduced by CLAUDE[24] and improved on by others[10]. A comparative study using histochemical techniques for the localization of phosphomonoesterase activity at an acid and alkaline p_H within normal and regenerating rat liver sections has been reported by others[17]. Centrifugation techniques have been applied in investigations of phosphomonoesterases within embryonic tissues[25] and rat liver[26, 27]. The series of experiments to be presented was designed to investigate the quantitative distribution of phosphomonoesterase activity within cellular fractions isolated by centrifugation techniques from normal and regenerating mouse hepatic tissues.

Phosphomonoesterase distribution within normal liver. Adult normal mouse liver was separated into a series of fractions by centrifugation procedures already described. The separated fractions were assayed for phosphomonoesterase activity at p_H 5.5 as well as at p_H 9.2.

In Table IV are presented the results of a representative experiment showing the relative distribution of phosphomonoesterase activity found at p_H 5.5 within isolated

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fractions from normal mouse liver. Attempts to accurately assay the levels of phosphomonoesterase activity at p_H 5.5 were complicated by the recognized labile nature of enzyme active at this p_H. In view of the length of time required to complete the fractionations (between 4–5 hours), some inactivation of enzyme was inevitable. In the experiment presented, 15% loss in enzyme was found within the total liver homogenate after 5 hours at near 0° C. From other experiments, however, it was found that the rates of enzyme inactivation within different samples were far from constant. A comparison of the enzyme activity remaining within the total homogenate after 5 hours with that of the separated fractions discloses a higher rate of inactivation of enzyme within the isolated fractions, resulting in a lack of complete recovery following the centrifugation procedure. The accelerated rate of enzyme inactivation associated with the fractionation procedure was attributed to a possible lack of adequate temperature control during the centrifugations or the removal of protective components by the separation procedure. The inability to demonstrate 100% recovery of enzyme within the separated fractions was not considered to be due to the assay procedure in view of the satisfactory agreement in each case between the duplicate analyses using two levels of tissue. From the results presented in Table IV, phosphomonoesterase active at p_H 5.5 was found to be distributed within all of the isolated liver fractions. Despite the inability to demonstrate the quantitative distribution of this enzyme, it was concluded from the results that the enzyme

TABLE IV

PHOSPHOMONOESTERASE ACTIVITY AT p_H 5.5 IN VARIOUS TISSUE FRACTIONS PREPARED FROM NORMAL FASTED MOUSE LIVER

Liver fraction	Time prior to enzyme assay (hours)	mg Nitrogen		mg P hydrolyzed/hr at 37° C and at p_H 5.5**			
		Total/g fresh liver	For enzyme assay	Found	Av./mg Nitrogen	Av./g original fresh liver	% of total remaining atfer 5 hrs
Total homogenate (A)	0	32.8	0.082 0.164	0.115 0.235	1.43	47.0	115
Total homogenate (B)	5	32.8	0.082 0.164	0.102 0.205	1.25	41.0	100
Nuclear fraction	5	5.10	0.128 0.256	0.072 0.142	0.55	2.86	7.0
Cytoplasmic fraction	5	28.0	0.080 0.160	0.100 0.203	1.27	35.5	86.6
Mitochondrial fraction	5	7.70	0.077 0.154	0.078 0.157	1.02	7.85	19.1
Microsomal fraction	5	3.08	0.0308 0.0616	0.076 0.151	2.45	7.55	18.4
Remaining supernatant	5	17.6	0.058 0.117	0.039 0.081	0.68	12.0	29.3

* Pool of livers from 5 mice utilized for the fractionation study. All tissues maintained near 0° C prior to, during and subsequent to the fractionations.

** Reaction mixtures contained in addition to tissue, a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* acetate buffer at p_H 5.5 in a final volume of 3 ml.

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was probably concentrated principally within the microsomal fraction and to the least extent within the nuclear fraction. The greatest total amount of enzyme, however, appeared to be present within the remaining supernatant liquor. The extent to which enzyme was extracted from each of the sedimented fractions could not of course be determined.

The same isolated tissue fractions utilized to investigate phosphomonoesterase levels at p_H 5.5 were also assayed for enzyme activity at p_H 9.2 (Table V). All procedures were identical with those adopted in the previous study. Phosphomonoesterase active at p_H 9.2 was found to be distributed within each of the isolated fractions. As previously shown, little loss in enzyme active at this p_H was evident over an appreciable length of time; consequently, good recovery within the separated fractions was achieved. The enzyme appeared to be concentrated within the cytoplasm rather than in the nuclear fraction. Each of the sedimented fractions contained relatively little enzyme, with approximately 80% of the enzyme present in the remaining supernatant liquor. These findings might suggest considerable extraction of enzyme from one or all of the sedimented fractions. The reliability of the assay method for enzyme is demonstrated by the good checks achieved on duplicate analyses using two levels of tissue concentration.

Phosphomonoesterase distribution within regenerating liver. Little difference in levels of phosphomonoesterase active at p_H 5.5 was previously demonstrated between normal adult and regenerating mouse hepatic tissues. Further experiments were performed

TABLE V

PHOSPHOMONOESTERASE ACTIVITY AT p_H 9.2 IN VARIOUS TISSUE FRACTIONS PREPARED FROM NORMAL FASTED MOUSE LIVER

Liver* fraction	mg Nitrogen			mg P hydrolyzed/hour at 37° C and p_H 9.2**			
	Total/g fresh liver	% of total	For enzyme assay	Found ($\cdot 10^{-3}$)	Av./mg Nitrogen	Av./g original fresh liver	% of total
Total homogenate	32.8	100	0.082 0.164	11.0 22.3	0.135	4.43	100
Nuclear fraction	5.10	15.5	0.128 0.256	11.6 23.3	0.090	0.46	10.4
Cytoplasmic fraction	28.0	85.4	0.080 0.160	11.7 22.9	0.145	4.05	91.4
Mitochondrial fraction	7.70	23.5	0.385 0.770	20.5 41.0	0.053	0.41	9.3
Microsomal fraction	3.08	9.39	0.154 0.308	12.2 25.0	0.081	0.25	5.6
Remaining supernatant	17.6	53.7	0.058 0.117	12.1 23.8	0.204	3.60	81.2

* Pool of livers from 5 mice utilized for the fractionation study. All tissues maintained near 0° C prior to, during and subsequent to the fractionations.

** Reaction mixtures contained in addition to tissue, a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* veronal buffer at p_H 9.2 in a final volume of 3 ml.

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attempting to demonstrate a possible alteration in the distribution of this enzyme within 65-hour regenerating liver. Although quantitative distribution values could not be attained because of unequal rates of enzyme inactivation again found within the isolated fractions, it was concluded from these experiments that little difference probably exists in the distribution of enzyme active at p_H 5.5 between normal and regenerating liver.

The distribution of phosphomonoesterase active at p_H 9.2 within 65 hours regenerating mouse liver was also investigated (Table VI). Some differences were found in the distribution of enzyme active at this p_H between normal and regenerating liver. An over-all increased level of enzyme was again found within the regenerating livers. Although little enzyme was evident in the nuclear fractions of normal liver, marked increases were found in this fraction separated from regenerating liver. The over-all increased level of enzyme in regenerating tissues was reflected by increases in each of the separated fractions. In addition to the observed increases in enzyme within these fractions, some differences in their quantitative distribution were apparent. The supernatant liquor contained less of the total enzyme than found for normal mouse liver, indicating either less extraction or a true difference in distribution. The submicroscopic particles (microsomes) contained a greater percentage of the total enzyme than found in normal liver. Nitrogen analyses on each of the isolated fractions indicated a greater percentage of the nitrogen recovered in both the nuclear and microsomal fractions isolated from the regenerating tissues.

TABLE VI

PHOSPHOMONOESTERASE ACTIVITY AT p_H 9.2 IN VARIOUS FRACTIONS PREPARED FROM 65 HOUR REGENERATING MOUSE LIVER

Liver* fraction	mg Nitrogen			mg P hydrolyzed/hour at 37° C and p_H 9.2**			
	Total/g fresh liver	% of total	For enzyme assay	Found ($\cdot 10^{-3}$)	Av./mg Nitrogen	Av./g original fresh liver	% of total
Total homogenate	26.6	100	0.0675 0.133	16.0 31.5	0.318	8.47	100
Nuclear fraction	5.01	18.8	0.0625 0.125	16.5 32.5	0.350	1.75	20.7
Cytoplasmic fraction	22.0	82.7	0.063 0.126	14.3 28.0	0.300	6.61	78.1
Mitochondrial fraction	6.02	22.6	0.150 0.301	14.9 30.0	0.133	0.80	9.5
Microsomal fraction	3.36	12.6	0.084 0.168	19.1 38.2	0.303	1.02	12.0
Remaining supernatant liquor	12.5	47.0	0.0417 0.0833	12.5 24.7	0.399	4.98	58.6

* Pool of livers from 8 mice utilized for the fractionation study. All tissues were maintained at near 0° C prior to, during and subsequent to the fractionations.

** Reaction mixtures contained in addition to tissue, a final concentration of 0.12 *M* Mg ions, 0.083 *M* substrate and 0.05 *M* veronal buffer at p_H 9.2 in a final volume of 3 ml.

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GENERAL DISCUSSION

Although specific attempts were not made to verify the presence of separate phosphomonoesterases active on phenylphosphate at p_H 5.5 and 9.2, some evidence was presented to support such a conclusion. Phosphomonoesterase active at p_H 5.5 as compared to 9.2 appeared to differ with respect to rate of inactivation as well as distribution and failed to show a difference in level during regenerative growth. The reaction rate of enzyme active at p_H 9.2 also appeared to be influenced to a greater extent by reaction products than enzyme active at p_H 5.5.

The increases observed in enzyme active at p_H 9.2 within regenerating tissues remains to be interpreted. In view of the recognized presence of enzyme active at this p_H within structures of the liver other than parenchymal cells[27,28] some difficulty is encountered in attempting to evaluate the experimental results. A concentration of enzyme active at p_H 9.2 was found within nuclear fractions isolated from regenerating liver. These findings are not in disagreement with histochemical evidence for increased levels of enzyme active at p_H 9.2 within nuclear structures of regenerating tissues[17,18,28].

From the centrifugation experiments, it was found that little phosphomonoesterase active at p_H 9.2 was recovered in the nuclear fractions from normal mouse liver. To what extent enzyme might have been extracted during the homogenization and centrifugation procedures could not be determined. In view of the relatively high concentrations of this enzyme found within the non-sedimentable fractions, some extraction of enzyme would appear to have occurred. DOUNCE[29] reported that normal rat liver cell nuclei isolated with dilute citric acid contained a greater concentration of phosphomonoesterase active in an alkaline medium than did the liver as a whole. Attempts to demonstrate a concentration of phosphomonoesterase active at p_H 9.2 within normal mouse liver cell nuclei isolated by the procedure utilized by DOUNCE proved unsuccessful. Nuclei isolated in this manner from normal mouse liver were found repeatedly to show less enzyme per mg nitrogen than the total liver, suggesting that some differences presumably exist in the nature of the nuclear membrane or in the levels of nuclear enzyme between the rat and mouse. Investigators[17] applying histochemical techniques have described the presence of a concentration of phosphomonoesterase active in an alkaline medium within rat liver cell nuclei. These findings, however, remain somewhat questionable in view of recent reports[28,30,31] describing certain difficulties associated with the true localization of enzyme by this technique.

It is apparent that the existing centrifugation methods are somewhat inadequate for the quantitative study of phosphomonoesterase localization within tissues. A further application of nonaqueous solvents[32,33] to the isolation of cytoplasmic constituents would appear to be fruitful.

SUMMARY

1. Accurate quantitative methods for the assay of phosphomonoesterase activity at p_H 5.5 and 9.2 within fresh liver homogenates were presented using phenylphosphate as the substrate.

2. The levels of phosphomonoesterase activity at p_H 5.5 and 9.2 were determined for normal adult and regenerating hepatic tissues of the mouse. Although increases in phosphomonoesterase activity were found at p_H 9.2 within the regenerating tissues, little differences were observed in enzyme levels at p_H 5.5.

3. Centrifugation techniques were incorporated to investigate the relative distribution of phosphomonoesterase activity at both p_H 5.5 and 9.2 within normal and regenerating hepatic tissues of

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the mouse. All separated fractions from normal adult mouse liver were found to contain phosphomonoesterase active at pH 5.5 and 9.2. Enzyme active at pH 5.5 appeared to be concentrated within the submicroscopic or microsomal fraction. Little concentration of enzyme active at pH 9.2 was found within any of the sedimented fractions of normal mouse liver; however, a concentration of this enzyme was present within the nuclear fractions isolated from regenerating liver. Extensive extraction of enzyme active at pH 9.2 from particulate components was suspected.

4. Differences in inactivation rates, sedimentability and relative alterations within regenerating liver were found between phosphomonoesterase active at pH 5.5 and 9.2 suggesting the presence of separate enzymes acting at these hydrogen ion concentrations.

RÉSUMÉ

1. Nous présentons des méthodes quantitatives exactes pour la détermination de l'activité phosphomonoestérasique à pH 5.5 et 9.2 dans des homogénats de foie frai, utilisant le phénylphosphate comme substrat.

2. Les niveaux d'activité phosphomonoestérasique à pH 5.5 et 9.2 ont été déterminés pour des tissus hépatiques, adultes normaux et en régénération, de souris. A pH 9.2 nous avons observé une augmentation de l'activité phosphomonoestérasique dans les tissus en régénération, par contre à pH 5.5, nous avons trouvé peu de différences entre les niveaux d'activité enzymatique.

3. Nous avons appliqué des techniques de centrifugation pour étudier la distribution relative de l'activité phosphomonoestérasique à pH 5.5 et 9.2 dans les tissus hépatiques normaux et en régénération de la souris. Nous avons trouvé que toutes les fractions isolées de foie de souris adulte normal contenaient de la phosphomonoestérase active à pH 5.5 et 9.2. L'enzyme active à pH 5.5 était concentrée dans la fraction submicroscopique ou microsomale. La concentration de l'enzyme active à pH 9.2 était faible dans toutes les fractions sédimentées de foie de souris normal; par contre cette enzyme était concentrée dans les fractions nucléaires isolées à partir de foie en régénération. Nous avons supposé qu'une extraction importante de l'enzyme active à pH 9.2 à partir de certaines composantes a eu lieu.

4. Les différences de vitesse d'inactivation, de sédimentabilité et d'altération relative dans le foie en régénération, trouvées entre la phosphomonoestérase active à pH 5.5 et celle active à 9.2 font prévoir la préence d'enzymes différentes agissant à ces concentrations d'ions hydrogène.

ZUSAMMENFASSUNG

1. Genaue quantitative Methoden zur Bestimmung der Phosphomonoesterase-Aktivität bei pH 5.5 und pH 9.2 in frischem Leberhomogenat unter Verwendung von Phenylphosphat als Substrat wurden beschrieben.

2. Die Höhe der Phosphomonoesterase-Aktivität bei pH 5.5 und 9.2 für normales und regenerierendes Lebergewebe der Maus wurde bestimmt. Obwohl bei pH 9.2 im regenerierenden Gewebe eine Zunahme der Phosphomonoesterase-Aktivität beobachtet wurde, konnten bei pH 5.5 nur wenig Unterschiede in der Höhe der Enzym-Aktivität festgestellt werden.

3. Zum Studium der relativen Verteilung der Phosphomonoesterase-Aktivität bei pH 5.5 und 9.2 in normalen und regenerierenden Lebergeweben der Maus wurden Zentrifugiermethoden eingeschaltet. Alle Fraktionen aus normaler Mäuseleber enthielten bei pH 5.5 und 9.2 aktive Phosphomonoesterase. Es zeigte sich, dass das Enzym, welches bei pH 5.5 aktiv ist in der submikroskopischen oder Mikrosom-Fraktion konzentriert war. Das bei pH 9.2 wirkende Enzym war in allen sedimentierten Fraktionen von normaler Mäuseleber wenig konzentriert; dagegen war dieses Enzym in den aus regenerierender Leber isolierten Kernfraktionen konzentriert. Es wurde angenommen, dass das bei pH 9.2 wirkende Enzym aus verschiedenen Bestandteilen stark extrahiert worden war.

4. Zwischen der bei pH 5.5 und der bei pH 9.2 wirkenden Phosphomonoesterase wurden Unterschiede festgestellt, die sich auf die Inaktivierungsgeschwindigkeit, die Sedimentierbarkeit und die Zerstörung innerhalb des regenerierenden Lebergewebes beziehen; auf Grund dieser Unterschiede wird auf das Vorhandensein zweier verschiedener Enzyme, welche bei den genannten Wasserstoffionen-Konzentrationen wirksam sind, geschlossen.

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